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The aim of this proposal was to examine the role of proteases and protease inhibitors in the regulation of growth factor release in breast cancer. To this end we report the cloning, sequencing and properties of a TGFα-releasing protease present in breast ductal epithelium from an MCF-7 breast cancer cell cDNA library. The deduced 321 amino acid open reading frame shows a high degree of homology with the cell-surface serine proteases enteropeptidase, prekalikrein, hepsin and prostasin. Northern analysis, showed the presence of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells and normal breast ductal epithelial cells. A GST fusion protein, constructed from a portion of the PCR-7 open reading frame cDNA sequence, was used to raise antibodies in rabbits. On western blot analysis, the antibody recognized a 33 kDa polypeptide in MCF-7 cells under both reducing and non-reducing conditions. This antibody inhibited TPA-induced TGFα release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera. Immunohistochemistry of paraffin sections from normal and cancerous human breast tissue showed that the PCR-7 protein was restricted to the surface of ductal epithelial cells where it co-localized with the epidermal growth factor receptor.

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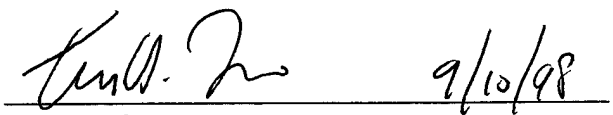

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## INTRODUCTION

The broad aim of this proposal is to test the hypothesis that the coordinate action of proteases and protease inhibitors are responsible for growth factor activation and release in the human breast cancer cell, and for the progression of breast cancer *in vivo*. One-third of all cases of advanced breast cancer are estrogen responsive, and recent epidemiological studies and studies using human breast cancer cells in culture strongly suggest a correlation between estrogens and the pathogenesis of breast cancer (Harris et al 1992). The mechanisms by which this occurs are not entirely clear. Certainly, estrogens have a direct effect on cell growth. In addition, they can stimulate the expression and release of a variety of polypeptide growth factors. It is highly likely that the tummorigenic effects of estrogens are due, at least in part, to the autocrine/paracrine action of these factors. Several of these polypeptides, including epidermal growth factor (EGF), and its analogs heregulin and transforming growth factor-α (TGFα) and the insulin-like growth factors (IGF-I and IGF-II), have been shown to require pericellular proteolysis for activation or release (Massagué and Pandiella 1993, Hooper et al 1997). To achieve homeostasis in a normal breast epithelial cell, levels of these pericellular growth factor activating proteases also must be regulated. We hypothesized that this was accomplished by the action of locally synthesized protease inhibitors. Thus an imbalance in the ratio between local levels of particular proteases and protease inhibitors could be responsible for increases in tumorigenic potential.

TGFα, a peptide structurally and functionally related to EGF, interacts with the EGF receptor (EGFR) and elicits a mitogenic response in a variety of cells (Lee et al 1995). TGFα expression occurs in normal breast tissue, breast tumors and breast cancer cells in culture and TGFα has been proposed to act as a major autocrine mediator of estrogen-stimulated growth in estrogen-dependent breast cancer cells (Harris et al 1992). Expression of the TGFα/EGFR pair have been shown to be associated with proliferation and angiogenesis in invasive breast cancer (De Jong et al 1998). TGFα is synthesized as part of a 20-22 kDa glycosylated, type I membrane protein precursor (proTGFα) which can be processed intracellularly and extracellularly by glycosylation and proteolysis to yield a family of polypeptides of from 6 to 17 Kda in size. (Massagué and Pandiella 1993, Baselga et al, 1996). Proteolytic processing occurs in two steps, the first results in cleavage between $Ala^{39}$ and $Val^{40}$ (Fig. 1). The second, occurring closer to the cell membrane, results in release of the 6 KDa mature TGFα peptide. While it is generally accepted that the second cleavage occurs between $Ala^{89}$ and $Val^{90}$, this does not rule out cleavage at another site (e.g between $Lys^{96}$ and $Lys^{97}$). Complete processing does not occur to the same extent in all tissues and it is certainly possible that shedding of TGFα from the cell surface may be accomplished by different proteases in different tissues (Hooper et al 1997, Arribas et al 1996). While all TGFα forms appear to possess some degree of biological activity, there is good evidence that particular biological actions may depend on the degree of proteolytic processing.

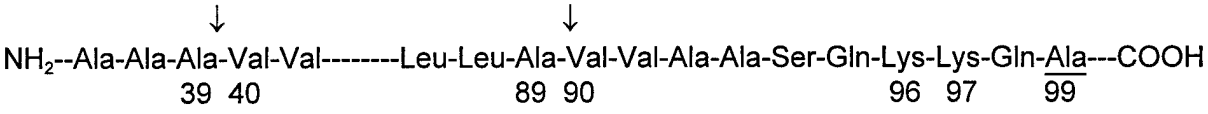



Figure 1. Cleavage sites in ProTGFα yielding mature 50 aa TGFα peptide. Arrows indicate the normal cleavage sites. $\underline{Ala}^{99}$ begins the hydrophobic membrane-spanning region.

Results from this laboratory have clearly demonstrated a relationship between tumorigenicity of MCF-7 human breast cancer cells (as measured by growth in soft agar), endogenous synthesis of the protease inhibitor $\alpha_1$-antitrypsin ($\alpha_1$-AT) and release of TGFα (Tamir et al 1990, Finlay et al 1993a,b). Growth in soft agar was blocked by $\alpha_1$-AT whether added to the tissue culture media or synthesized by the tumor cell itself. A useful tool in these studies was a new MCF-7 cell subline, producing 10-fold higher levels of $\alpha_1$-AT than its parental cell line, constructed by stable transfection with an $\alpha_1$-AT cDNA (Yavelow et al 1997). Growth in soft agar and release of TGFα was decreased in cells transfected with

the $\alpha_1$-AT cDNA when compared to cells transfected with vector alone. Consistent with the above we had identified a serine protease with elastase-like activity, capable of forming a stable complex with $\alpha_1$-AT, on the MCF-7 cell surface.

Our specific aims as stated in our grant application were to:

1. To identify and clone the growth-modulating pericellular proteases from MCF-7 cells, particularly, the elastase-like enzyme(s) that are able to effect the release of TGF$\alpha$ from the tumor cell surface.
2. To show that the ability of MCF-7 sublines to form colonies in soft agar and tumors in nude mice is a function of their expression of $\alpha_1$-AT and specific pericellular proteases.
3. To extend our observations relating to TGF$\alpha$ release/activation made in MCF-7 human breast cancer cells in culture to other breast cancer cell lines, and to normal and malignant human breast tissue.
4. To test the hypothesis that the pericellular protease:protease inhibitor ratio responsible for TGF$\alpha$ release can be regulated by ST-3 and other potential effectors such as antiestrogens, phorbol esters and SEC receptor agonists. This information may provide insight into mechanisms by which protease and protease inhibitor levels may be independently controlled.

To accomplish the above specific aims, we proposed to carry out the sequence of studies described in our Statement of Work. While the project has not proceeded in exactly the order as originally anticipated, I believe that we are fairly well on track. Our progress is outlined below.

| YEAR | STATUS OF PROJECTED STUDY |
|---|---|
| 1-2 | Isolate, characterize and clone proteases from MCF-7 cells. This phase of the work has been completed and a manuscript describing the cloning and properties of the TGF$\alpha$ cleavage protease has been submitted for publication. |
| 1-3 | Compare production of protease and protease inhibitors by MCF-7 cell sublines with their ability to form colonies in soft agar and cause tumor formation in nude mice. Because of difficulties in generating the MCF-7 cell clones hyper-producing pro-TGF$\alpha$, PCR-7 protease, and $\alpha_1$-antitrypsin-(Pittsburgh), this project is a little behind schedule. However, this phase of the work should be completed by the end of the year. |
| 1-3 | Extend our observations relating to TGF$\alpha$ release/activation made in MCF-7 human breast cancer cells in culture to other breast cancer cell lines, and to normal and malignant human breast tissue. We have demonstrated production of PCR-7 protease in normal and malignant breast epithelial tissue and anticipate looking at TGF$\alpha$ shedding in other breast cancer cell lines and in breast epithelial tissue over the next several months. Breast cancer cell studies using the non-transformed cell line MCF-10A, the ER-negative cell lines BT-20 and T47D and a second ER-positive cell line, ZR-65-1 are being carried out in collaboration with John Yavelow of Rider University. |
| 2-4 | Examine production and localization of proteases, protease inhibitors, growth factor receptors and sites of growth factor activation in normal and malignant breast tissue. In collaboration with Dr. Helen Feiner (Department of Anatomic Pathology) we have begun to look at histochemical localization of PCR-7 protease, EGF receptor and ProTGF$\alpha$ in normal and maligmnant breast tissue. |
| 1-4 | Test the hypothesis that the pericellular protease:protease inhibitor ratio responsible for TGF$\alpha$ release can be regulated by ST-3 and other potential effectors Identify potential modulators of protease inhibitor: protease ratio and growth factor activation/release in breast cancer cells and in ductal epithelium from human breast. |

## METHODS AND MATERIALS

### *1. Construction of MCF-7 Cell Sublines Demonstrating Stabile Over-expression of $\alpha_1$-Antitrypsin Pittsburgh ($\alpha_1$- ATp).*

The $\alpha_1$-ATp mutant in the vector pMT3 was obtained from Dr. A. Rehemtulla of the University of Michigan Medical Center. The Eco RI-Sal I cDNA fragment was excised from the vector and blunt ended. After addition of HIND III linkers, the fragment was subcloned into the eukaryotic expression vector pRC/CMV (Invitrogen) which contains the gene encoding for neomycin resistance (neo$^r$). This is the same vector that was used to construct MCF-7 cell sublines over expressing $\alpha_1$-AT. The orientation of the insert with respect to the cytomegalovirus promoter was determined by restriction mapping of the subclones, and plasmid containing the insert in the sense orientation was amplified for transfection. Initially, MCF-7 cells were transfected by electroporation since this method was supposed to be more efficient. Although Northern analysis showed a slight increase in $\alpha_1$- ATp mRNA in some putative transfected colonies, none demonstrated an increase in secreted $\alpha_1$-ATp protein as measured by Western blotting. This might have been due to cells escaping selection because the plating density was too high. Subsequently we repeated the transfection using both the calcium phosphate method and the lipid transfection technique (LipoTAXI mammalian transfection kit, Stratagene). After transfection the cells were plated at low density and cultured for approximately 14 days in medium containing 550 μg/ml Geneticin (G418). The media was changed every 4-5 days to remove dead cells. 21 distinct G418 resistant colonies were trypsinized using cloning rings and transferred to 15 mm wells. 10 of these colonies were selected for expansion and analysis. Two of the first seven colonies subjected to Northern analysis demonstrated elevated message levels indicating successful transfection (**Fig. 2A**). One clone (lane 2) over-expressed a much larger mRNA (approximately 4 Kb). Subsequently, the cells will be labeled with [$^{35}$S]-methionine and the media immunoprecipitated with antibody to $\alpha_1$-ATantitrypsin to determine expression of protein. Our finding that the PCR-7 protease (see below) may have an Arg/Lys specificty pocket adds considerable importance to the construction of this cell line as $\alpha_1$-ATp inhibits trypsin-like but not elastase-like enzymes while $\alpha_1$-AT inhibits both classes of proteases.

### *2. Construction of MCF-7 Cell Sublines Demonstrating Stabile Over-expression of PCR-7 protease.*

The cloning vector pSport 1 containing the PCR-7 protease cDNA was digested with Sal I. After filling in the ends with DNA polymerase 1 (Klenow) and ligating Hind III linkers the plasmid was digested with Hind III and Not I. This fragment was purified and ligated to the Hind III – Not I site of the eukaryotic expression vector pRC/CMV (see above). This strategy ensured that the insert is in the correct orientation for expression. MCF-7 cells were transfected with this construct by both the calcium phosphate and lipid transfection technique. The cells were treated as above and presently 10 clones are being amplified and analyzed. Three of the first six colonies subjected to Northern blot analysis demonstrated elevated message levels indicating successful transfection (**Fig. 2B**). With these cells both the media and cell lysates of metabolically labeled cells will be immunoprecipitated with antibody to PCR-7.

It should be noted that no significant difference in efficiency between the calcium phosphate and the lipid transfection method was observed. However, in the case of $\alpha_1$-Atp, the cells prepared by the lipid transfection technique were plated too heavily to permit selection of distinct colonies so all clones were selected from transfectants prepared by the calcium phosphate method. For the PCR-7 clones, transfectants prepared by both methods were selected.

### *3. Construction of MCF-7 Cell Sublines Demonstrating Stabile Over-expression of proTGFα.*

Because of the low endogenous expression of proTGFα and corresponding low levels of TGFα shedding in wild-type MCF-7 cells, obtaining reliable results in which TGFα release is used as an endpoint is difficult by standard assays. Even when using large numbers of cells (i.e. T-75 flasks), TGFα in the media must be concentrated either by ultra-filtration or immunoprecipitation prior to assay

either by ELISA or western blot analysis. To simply routine assays we will construct an MCF-7 cell subline over expressing proTGFα. This will be accomplished using techniques similar to those used for the construction cell lines over expressing $\alpha_1$-AT, PCR-7 protease and $\alpha_1$-AT-(Pittsburgh). To this end we will subclone a 925 bp proTGFα cDNA (phTGF1-10-925, ATCC) into the vector pRC/CMV. The Eco RI fragment will be blunt ended and after addition of HIND III linkers ligated into the expression vector. The orientation of the insert will be determined by restriction mapping.

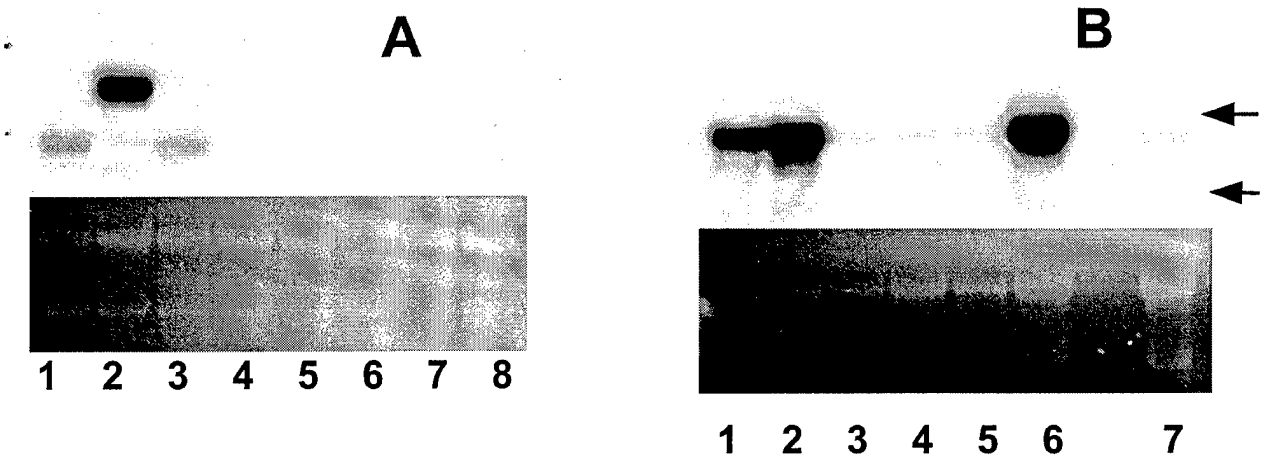



**Figure 2. Northern blot analysis of MCF-7 clones over-expressing $\alpha_1$-ATp and PCR-7 protease.** Individual colonies were selected and expanded. Aliquots of expanded colonies were grown to confluence in wells of 6-well plates and total RNA was isolated with Genosys RNA Isolator. Appoximately 8 μg of total RNA from each sample was electrophoresed on 1.2% agarose gels, transferred to nylon membranes and hybridized with the appropriate $^{32}$P-labeled cDNA probe. The bottom half of each figure shows a image of the ethidium bromide stained gel to estimate the degree of loading **(A) $\alpha_1$-ATp.** The probe used was a 405 bp PCR-generated fragment of the human $\alpha_1$-AT cDNA comprising nucleotides 234-639. Lane 8 contained 8 μg of RNA from wild-type non-transfected MCF-7 cells. **(B) PCR-7 Protease.** The probe used to screen the blot was a 300 bp PCR-generated fragment of the full-length cDNA. Lane 7 contained 8 μg of RNA from wild-type non-transfected MCF-7 cells. Neither of the probes recognized the message for antibiotic resistance coded for by the vector.

***4. Effect of Protease Inhibitors on Growth of MCF-7 Cells in Soft Agar***

Soft agar transformation assays of MCF-7 cells were carried out essentially as described previously (Tamir et al 1990). Cells, maintained for 24-48 hr. in RPMI-1640 media containing 10% charcoal-treated FBS, were plated in RPMI-1640 media containing 1% ultra low melting agarose (SeaPrep, FMC BioProducts), 10% charcoal-treated, TIC-depleted FBS, penicillin, streptomycin and glutamine with and without E2 ($10^{-8}$ M) over a bottom layer of 2% of the same agarose. Cells were plated at a density of $10^4$ cells per well in 30 mm tissue culture dishes and incubated at 37 C in an atmosphere containing 5% $CO_2$. After 14 days, the plates were stained for 24 hr. with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride and the number of colonies per well (aggregates of more than 40 cells, >100 μm) were counted manually.

In preliminary studies, cells were incubated with and without $\alpha_1$-AT (50 μg/ml), the serine protease inhibitor 3,4 dichloroisocoumarin (DCI, $10^{-4}$ M) and the metalloprotease inhibitor BB-3103 (British Biotech, $10^{-4}$ M). As we had previously demonstrated, E2 stimulated colony formation. In addition, all three protease inhibitors significantly reduced colony formation by wild-type MCF-7 cells under these conditions. These, not particularly novel results, confirm that in our system both metallo- and seine proteases play a role in tumorigenesis.

## RESULTS AND DISCUSSION

### *1. Cloning of the TGFα Releasing Enzyme*

As we reported last year, degenerate oligonucleotides based on the conserved sequences about the $his^{57}$, $asp^{102}$ and $ser^{195}$ residues in mammalian serine proteases were used to clone a potential TGFα-releasing serine protease from an MCF-7 breast cancer cell cDNA library. In the initial step, a series of approximately 500 bp fragments between the $his^{57}$ and $ser^{195}$ sites were amplified by PCR. The amplified sequences were then cloned into a PCR cloning vector, which was used to construct a mini-cDNA library. Clones from the mini library were selected by Southern blotting using a $^{32}P$-labeled degenerate oligonucleotide probe based on the sequence about $asp^{102}$. Several positive clones were sequenced. The 460 bp sequence of one strongly hybridizing clone, designated PCR-7, showed a high degree of homology to known serine proteases (64% identity in a 220 bp region at the 3' terminus to human trypsinogen-B and a 60% identity in a 120 bp region around the 5' terminus to human pancreatic protease). Northern blot analysis, using a PCR-7 cDNA probe, showed the presence of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells. Similar mRNA species were found in normal breast tissue, trophoblast, and proliferative phase uterine glandular epithelium but not in human U373MG glioma or HepG2 hepatoma cells.

Two cDNAs of approximately 3.8 and 3.2 kb containing the PCR-7 sequence were cloned from a second MCF-7 cell cDNA library using the PCR-7 sequence as a probe. Except for minor differences in the 3' sequence, the two clones appear to be identical where they overlap. Both clones hybridized to the same size mRNA as did the PCR-7 sequence used to screen the library. Last year we reported the sequence of approximately 2000 bp (plus an additional 500 bp at the 5' end) of the larger clone. We have now sequenced the entire 3271 bp cDNA at least twice and have resolved all apparent ambiguities (**Fig. 3**). The sequence has a 965 b open reading frame (bases 1766-2731) and an approximately 500 b 3' sequence containing the poly adenylation site. The deduced amino acid sequence from the open reading frame contains the $His^{57}$, $asp^{102}$ and $Ser^{195}$ motifs characteristic of serine proteases (amino acid residues 119-124, 176-180 and 267-272 respectively) and is compatable with the S1 family of the SA clan of serine-type petidases (Rawlings and Barrett, 1994). The PCR-7 protease may cleave peptide chains after Lys or Arg residues as it, like trypsin, hepsin and enterokinase contains an $Asp^{266}$ at the base of the specificity pocket (S1 subsite) (**Fig. 4**). Elastase and chymotrypsin-like enzymes have cysteine and serine residues, respectively, at this site.

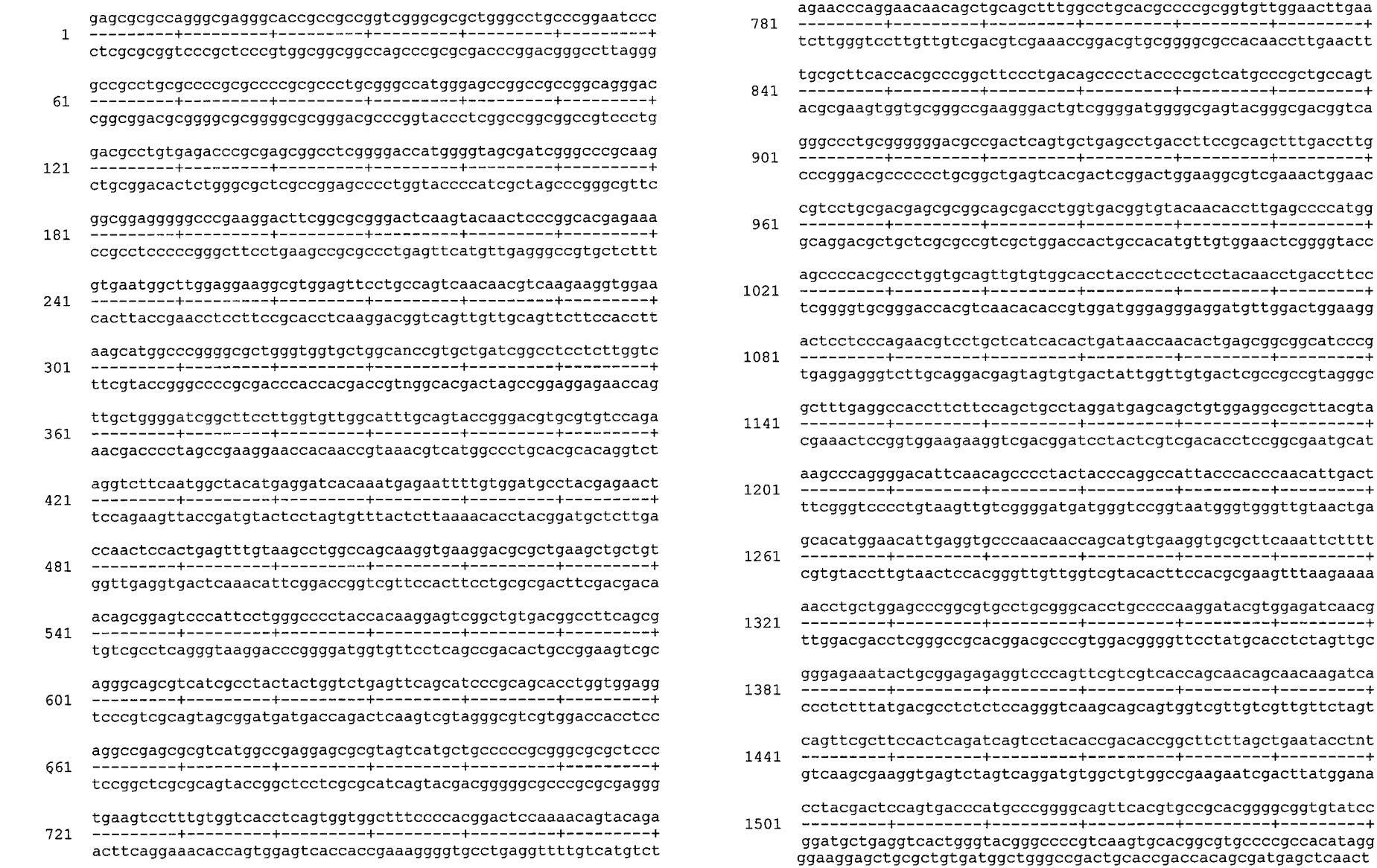
Using BLAST, a 45-55% identity at the amino acid level was found between the PCR-7 protease sequence and the serine proteases (or their zymogens) enterokinase precursor (Kitamoto et al 1995), hepsin (Leytus et al 1988), prekalikrein (Chung et al 1986), TMPRSS2 protease (Paolino-Giacobino, et al 1997), prostasin (Yu et al 1995) and drosophila protease stubble (Appel et al 1993) (**Table I**). Significantly, enterokinase precursor, hepsin, prekalikrein, and prostasin are all cell membrane-bound proteases. A high degree of homology was found at the nucleotide level with another cell surface protease, hepatocyte growth factor activator (Miyazawa et al 1993). The PCR-7 protease shows two potential membrane-anchoring N-myristoylation sites between deduced residues 43 and 54, three possible phosphorylation motifs at residues 6-9, 19-22 and 86-89 and a potential Asn glycosylation site at residues 238-241. These observations are consistent with the PCR-7 protease being a type II membrane protein. Enterokinase is activated by cleavage into disulfide-linked heavy and light chains by duodenase, a recently identified chymotrypsin-like serine protease (Sokolova et al 1998). A sequence very similar to the enterokinase cleavage site, ITPKIVGG, is present at residues 82-86 in the PCR-7 enzyme.

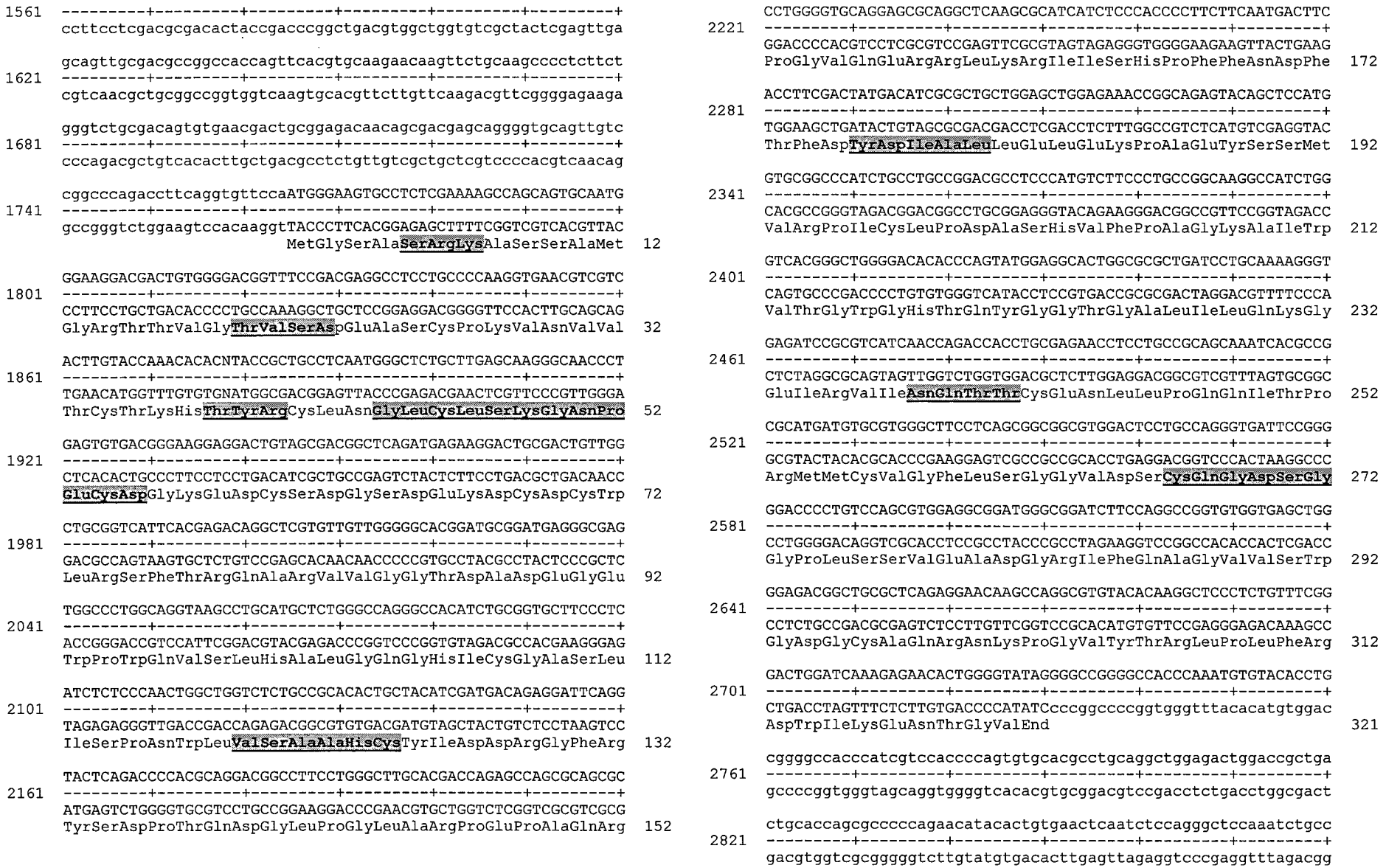
***2: Production of Antibodies to the TGFα Clevage Enzyme***

. The 460 bp PCR-7 cDNA was also ligated into the glutathione S-transferase (GST) gene fusion vector, pGEX-4T-1. This construct and the parental vector were used to transform E. coli and fusion protein expression was induced with IPTG. On SDS PAGE, 3 of 6 recombinant vectors displayed a prominent band of about 40 kDa representing the GST- PCR-7 fusion protein while the parental vector produced a prominent 29 kDa band characteristic of GST alone. Large scale cultures of bacteria containing the GST- PCR-7 fusion protein vector were prepared and substantial amounts of the GST- PCR-7 fusion protein was isolated. The fusion protein was used to raise antibodies in two rabbits. On western blot analysis, the antibody recognized a 33 kDa polypeptide in trophoblast and MCF-7 cells under both reducing and non-reducing conditions. This antibody inhibited TPA-induced TGFα release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera. Preliminary studies in last years report suggesting that this antibody is able to inhibit TPA-induced TGFα release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera were confirmed (**Fig. 5**).

MCF-7 cells were surface labeled with [$^3$H]-diisopropylfluorophosphate (DIFP) and immunoprecipitated with antibody to the GST-PCR-7 fusion protein. When total proteins were analyzed by SDS-polyacrylamide gel electrophoresis two labeled bands of approximately 32 and 70 kDa respectively were detected. Pretreatment of the cells with O-tetradecanoyl-phorbol-13-acetate (TPA), which has previously been shown to stimulate the release of TGFα in MCF-7 cells in the presence of estradiol (Yavelow et al, 1997), had no apparent effect on the intensity of either band. Our failure to immunoprecipitate the $^3$H-DIFP labeled PCR-7 protease in this preliminary experiment is disturbing as it might indicate that the protein is not on the cell surface. However, it is likely that the labeled bands represent mixtures of several cell surface serine proteases of similar sizes, of which the PCR-7 protease is only a very small fraction.

## Figure 3. Complete PCR-7 cDNA Sequence (3/98)





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GCGTACTACACGCACCCGAAGGAGTCGCCGCCGCACCTGAGGACGGTCCCACTAAGGCCC
ArgMetMetCysValGlyPheLeuSerGlyGlyValAspSerCysGlnGlyAspSerGly 272
GGACCCCTGTCCAGCGTGGAGGCGGATGGGCGGATCTTCCAGGCCGGTGTGGTGAGCTGG 2581
CCTGGGGACAGGTCGCACCTCCGCCTACCCGCCTAGAAGGTCCGGCCACACCACTCGACC
GlyProLeuSerSerValGluAlaAspGlyArgIlePheGlnAlaGlyValValSerTrp 292
GGAGACGGCTGCGCTCAGAGGAACAAGCCAGGCGTGTACACAAGGCTCCCTCTGTTTCGG 2641
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GlyAspGlyCysAlaGlnArgAsnLysProGlyValTyrThrArgLeuProLeuPheArg 312
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CTGACCTAGTTTCTCTTGTGACCCCATATCcccggccccggtgggtttacacatgtggac
AspTrpIleLysGluAsnThrGlyValEnd 321
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gccccggtgggtagcaggtggggtcacacgtgcggacgtccgacctctgacctggcgact
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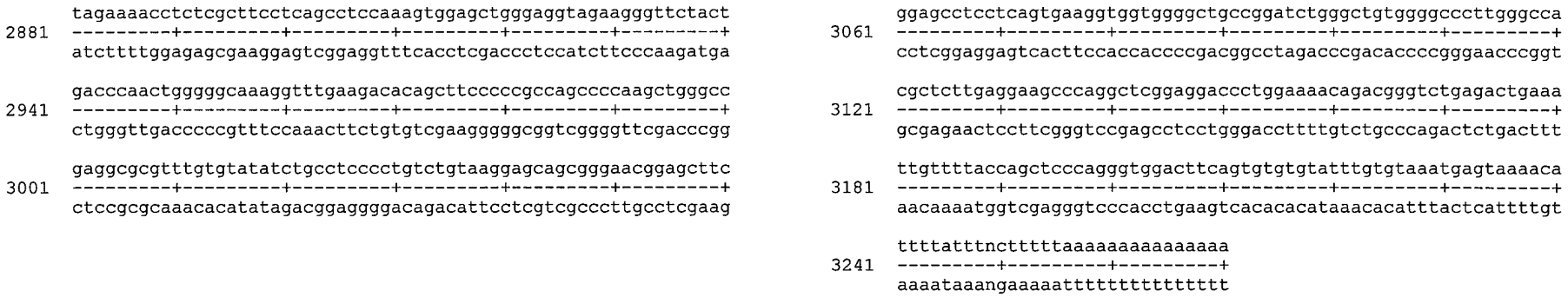


## Table I. Comparison of the Ser195 Catalytic Regions in PCR-7 and Related Serine Proteases

| | 266 | 272 |
|---|---|---|
| PCR-7 protease | GGV**D**SCQGD**S**GGPL | |
| Enterokinase Human (p98073) | GGI**D**SCQGD**S**GGPL | |
| Hepsin Human (p05981) | GGI**D**ACQGD**S**GGPF | |
| Trypsinogen 1 Human (p07477) | GGK**D**SCQGD**S**GGP | |
| Chymotrypsinogen Bovine (p00766) | SGV**S**SCMGD**S**GGPL | |
| Elastase Human (p08218) | GVI**C**TCNGD**S**GGPL | |

GenBank accession numbers appear in brackets

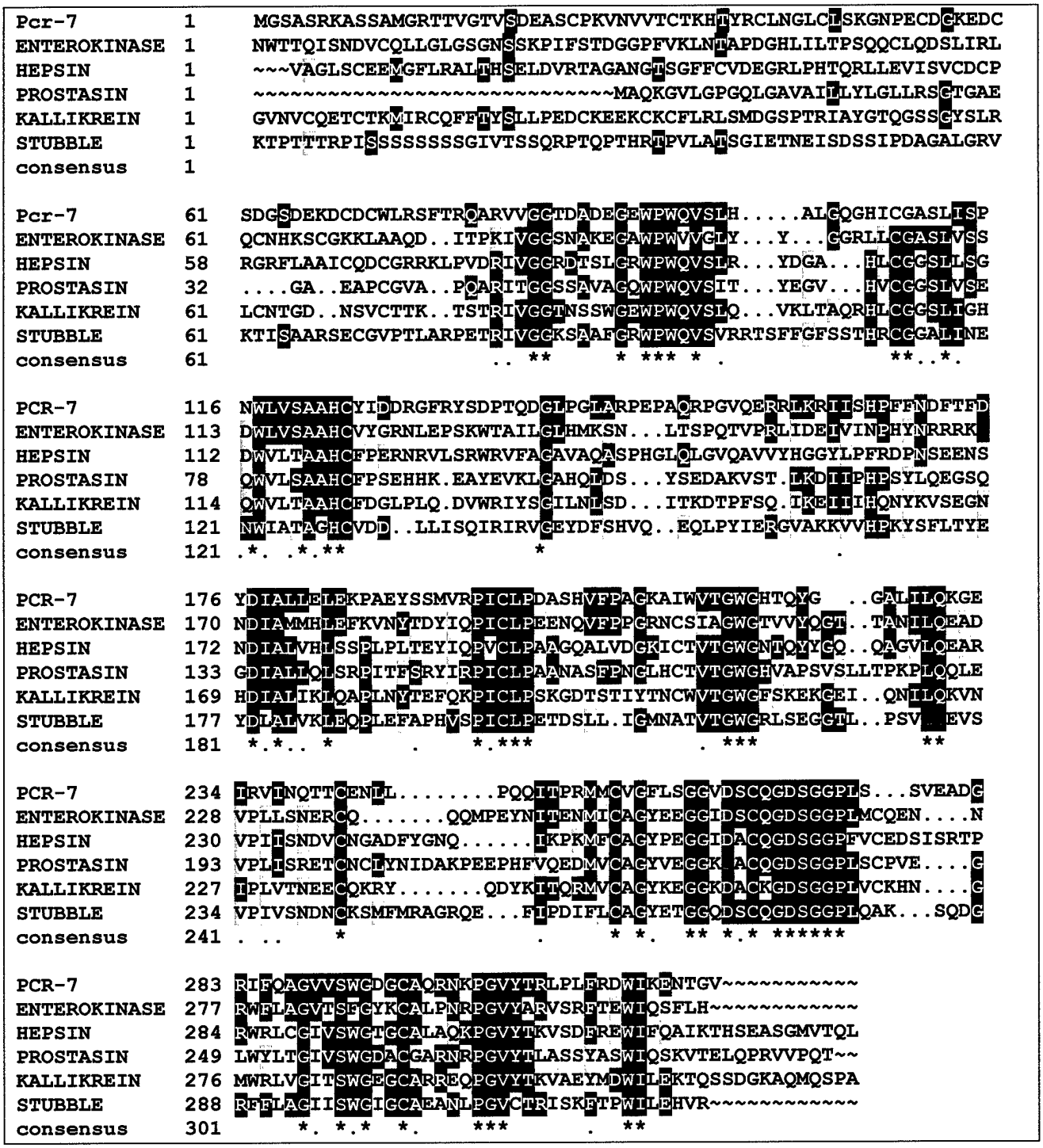


**Figure 4. Consensus Sequence of PCR-7 and Related Serine Proteases**

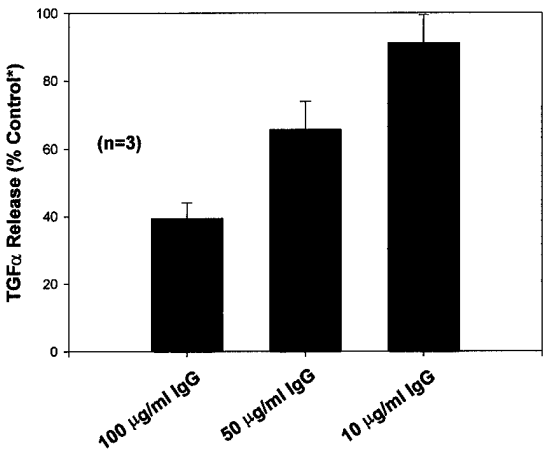


**Figure 5. Antibody to PCR-7 fusion protein blocks release of TGFα from MCF-7 cells.** MCF-7 (ML) cells were maintained in phenol red - free DMEM containing 10% FBS. Estradiol ($10^{-8}$ M) was added for 24 hours. Cells were then washed twice with serum-free media and allowed to condition serum-free DMEM containing estradiol and IgG for 30 min. TPA (50 ng/ml) was added and the cells were incubated for an additional 4 hours. Conditioned media were concentrated on $C_{18}$ SEP-columns equilibrated with 10% acetonitrile-0.1% TFA eluting with 40% acetonitrile-0.1% TFA. Samples were taken to dryness and resuspended in PBS. TGFα levels were determined using a TGFα ELISA assay (Oncogene Science) according to the manufacturer's instructions. Results are reported as the percentage of TGFα released in the presence of antibody (IgG) to the PCR-7-GST fusion protein as compared to the same level of pre-immune IgG.

### *3. PCR-7 Protein is Conserved in Mammalian Species.*

Southern analysis, using a PCR-7 cDNA probe, indicated the presence of homologous sequences in human, mouse, baboon, rat and rabbit but not in chicken or drosophila genomic DNA (**Fig. 6**). Using this same probe, expression of an mRNA specie similar in size to that found in MCF-7 cells, human breast and uterine epithelium and in trophoblast was demonstrated in mouse testes, kidney, ovary and uterus (**Fig. 7**). RNA from mouse breast or placenta was not tested.

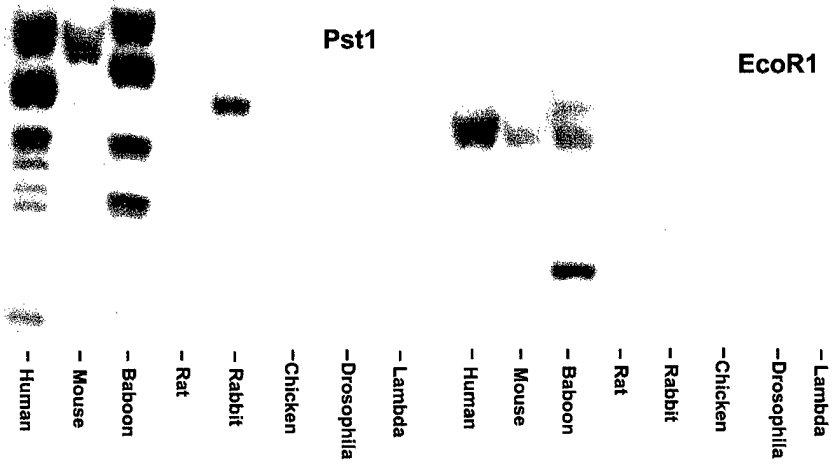


**Figure 6. Southern Blot Analysis for Related PCR-7 Protein Genes in Genomic DNA from Various Species.** Genomic DNA (15 µg) isolated from the species indicated, was digested with either Pst1 or EcoRI and electrophoresed on a 0.8% agarose gels. After alkaline denaturation and neutralization, the DNA was transferred to GeneScreen nylon membrane, fixed by baking and hybridized with a $^{32}$P-labeled PCR-7 probe.

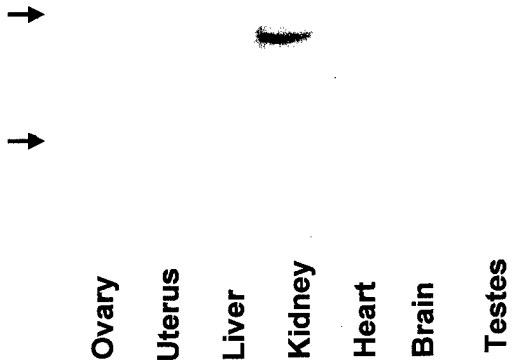


**Figure 7. Northern Blot Analysis of mRNA From Various Mouse Tissues.** The blot was screened with a $^{32}$P-labeled 300 bp fragment of the full-length PCR-7 protease cDNA generated by PCR.

### *4. Immunohistochemical Localization of PCR-7 Protein in MCF-7 Cells and Normal and Malignant Breast Tissue*

IgG from PCR-7 antisera was purified by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-cellulose. Using this IgG fraction, the PCR-7 protein was immuohistochemically localized to the cell surface of formalin-fixed MCF-7 cells (**Fig. 8**). We have begun to examine paraffin sections of tissues from reduction mammoplasty and malignant and adjacent 'normal' breast tissue histologically for the presence of EGFR, proTGF$\alpha$, $\alpha_1$-AT and the PCR-7 protease. These studies are being carried out with the cooperation of Helen Feiner, M.D. of the Department of Pathology, who has provided us with the samples and who has assisted us with the histology. In preliminary studies we have examined tissues from both normal regions and severe cancers. Once we have documented the differences between normal tissue and severe cancers, we will look at intermediate forms such as carcinoma *in situ*..Consistent with our findings in MCF-7 cells, PCR-7 co-localized with EGFr to the surfaces of breast epithelial cells (**Fig. 9**). In a highly malignant breast cancer (**Fig. 10**), staining for PCR-7 appeared to be reduced, a not uncommon occurrence when normal control mechanisms are lost.

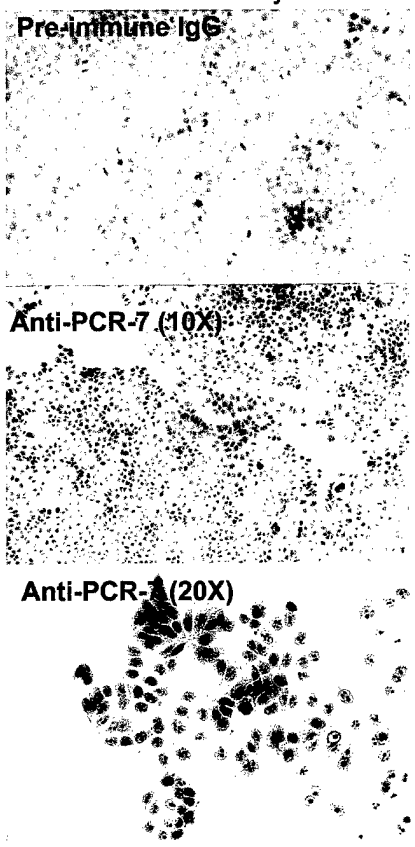


**Figure 8. Immunohistochemical localization of the PCR-7 protease to the surface of MCF-7 cells (left).** MCF-7 cells were cultured in plastic chamber slides to confluency. After 2 PBS washes, cells were fixed with 4 % para-formaldehyde and rehydrated. Endogenous peroxidase was quenched for 5 min using 5 % hydrogen peroxide in 100 % methanol. After 2 PBS washes, slides were incubated overnight at 4 C using 115 $\mu$g/ml of rabbit polyclonal anti-PCR7 antibody or the same amount of pre-immune IgG. Treatment with antirabbit peroxidase conjugate and color development with diaminobenzidine were carried out using the Vectastain ABC kit. Samples were counterstained with hematoxylin.

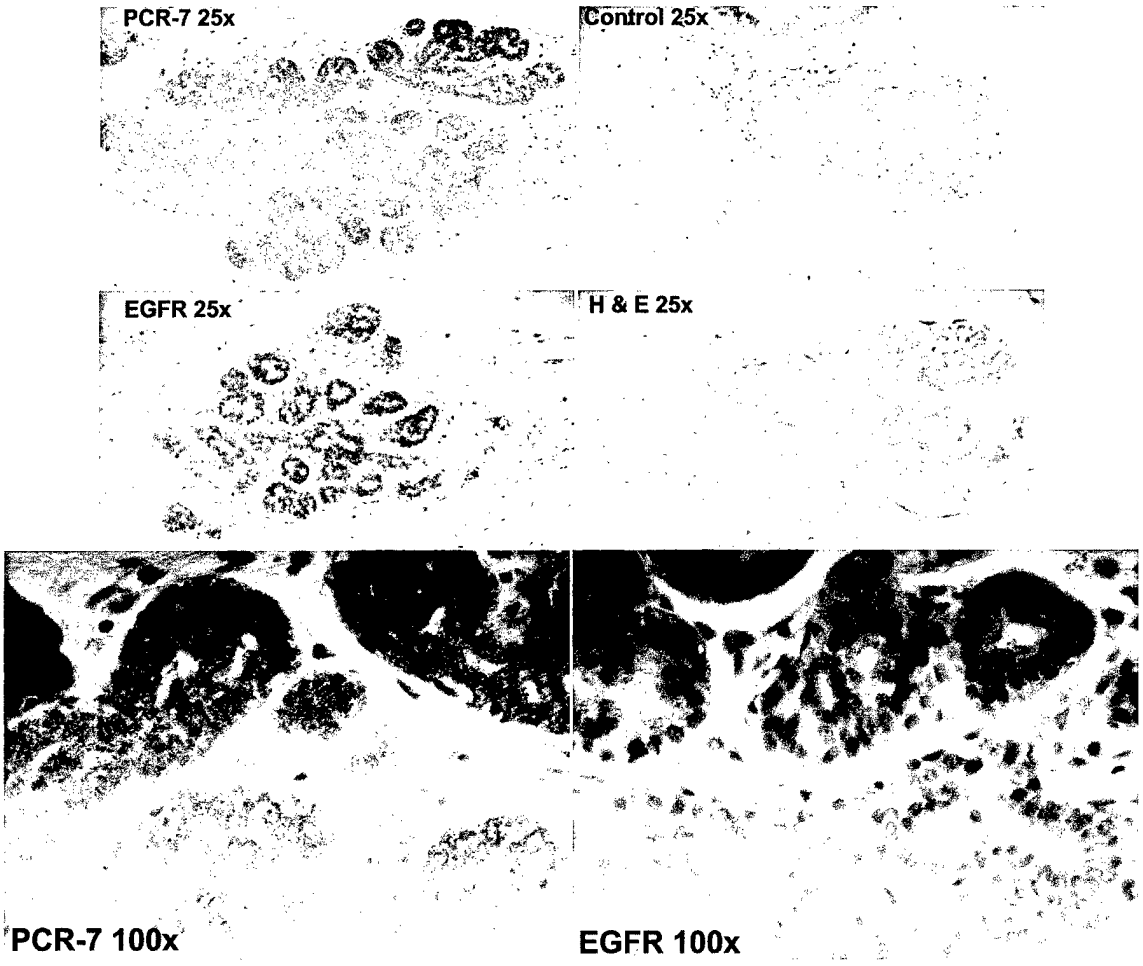


**Figure 9. Immunohistochemical co-localization of PCR-7 protease and EGFR to outer surfaces of breast epithelial cells.** Five μM paraffin-embedded tissue sections from a normal region of a benign breast tumor were applied to poly-L-lysine treated glass slides and deparaffinized for 2 h at 60 C before xylene and ethanol rehydration. Endogenous peroxidase was quenched for 5 min using 5 % hydrogen peroxide in 100 % methanol. After 2 PBS washes slides were incubated overnight at 4 C using 115 μg/ml of rabbit polyclonal anti-PCR7 antibody, the same amount of pre-immune IgG or 2 μg/ml of mouse monoclonal anti-human EGF-R antibody. Treatment with antirabbit or antimouse peroxidase conjugate and color development with diaminobenzidine were carried out using the Vectastain ABC kit. Samples were counterstained with hematoxylin and one set of tissues was stained with hematoxylin and eosin for morphology.

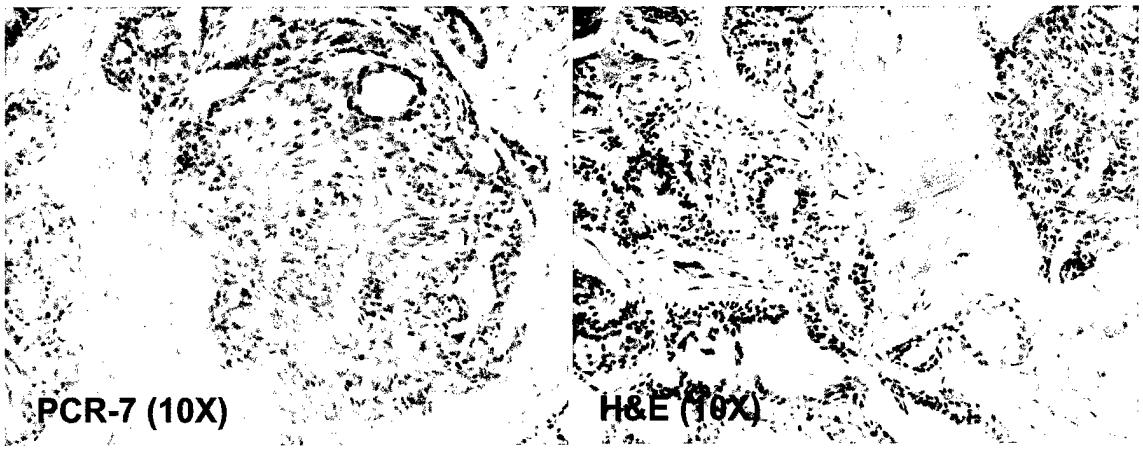


**Figure 10. Immunohistiochemical localization of PCR-7 protease in malignant breast carcinoma (8031).** Five μM paraffin-embedded tissue sections were applied to poly-L-lysine treated glass slides and deparaffinized for 2 h at 60 C before xylene and ethanol rehydration. Endogenous peroxidase was quenched for 5 min using 5 % hydrogen peroxide in 100 % methanol. After 2 PBS washes, the slides were incubated overnight at 4 C using 115 μg/ml of rabbit polyclonal anti-PCR7 antibody. Treatment with antirabbit peroxidase conjugate and color development with diaminobenzidine were carried out using the Vectastain ABC kit. Samples were counterstained with hematoxylin and one set of tissues was stained with hematoxylin and eosin for morphology.

## CONCLUSSIONS

Degenerate oligonucleotides based on the conserved sequences about the $his^{57}$ and $ser^{195}$ residues in mammalian serine proteases were used to clone a novel 3270 bp sequence (PCR-7) from an MCF-7 breast cancer cell cDNA library. The deduced 321 amino acid open reading frame shows a high degree of homology with the cell-surface serine proteases enteropeptidase, prekalikrein, hepsin and prostasin. Southern analysis, using a PCR-7 cDNA probe, indicated the presence of homologous sequences in monkey, mouse, and rabbit but not in chicken or drosophila DNA. Northern analysis, using this same probe, showed the presence of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells, normal breast ductal epithelial cells, trophoblast, and uterine glandular epithelium but not in human U373MG glioma or HepG2 hepatoma cells. Using this probe, expression of a similar-sized mRNA was demonstrated in mouse testes, kidney, ovary and uterus. In MCF-7 cells, expression of the PCR-7 mRNA was not significantly stimulated by estradiol or TGF$\alpha$, but was stimulated by IL-6 (200%) and IL-1 (300%). A GST fusion protein, constructed from a portion of the PCR-7 open reading frame cDNA sequence, was used to raise antibodies in rabbits. On western blot analysis, the antibody (IgG fraction) recognized a 33 kDa polypeptide in trophoblast and MCF-7 cells under both reducing and non-reducing conditions. This antibody inhibited TPA-induced TGF$\alpha$ release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera and blocked MCF-7 cell proliferation as measured by [$^3$H]-thymidine incorporation. Immunohistochemistry of paraffin sections from normal human breast tissue showed that the PCR-7 protein was restricted to the surface of ductal epithelial cells where it co-localized with the epidermal growth factor receptor.

## REFERENCES


Appel, L.F., Prout, M., Abu-Shumays, R., Hammonds, A., Garbe, J.C., Fristrom, D.K. and Fristrom, J.W. 1993 Proc Natl Acad Sci USA 90:4937-4941.

Arribas, J., Coodly, L., Vollmer, P. Kishimoto, T. K., Rose-John, S. and Massagué, J. 1996 Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. J Biol Chem 271: 11376-11382.

Baselga, J., Mendelsohn, J., Kim, Y-M. and Pandiella, A. 1996 Autocrine regulation of membrane transforming growth factor-$\alpha$ cleavage. J Biol Chem 271:3279-3284.

Chung, D.W., Fujikawa,K., McMullen,B.A. and Davie,E.W. (1986) Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. Biochemistry 25: 2410-2417.

De Jong, J.S., Van Diest, P.J., Van der Valk, P. and Baak, J.P. 1998 Expression of growth factors, growth inhibiting factors and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol 184:53-57.

Finlay, T.H, Tamir, S., Kadner, S.S., Yavelow, J. and Levitz, M. 1993a $\alpha_1$-Antitrypsin and Anchorage-Independent Growth of MCF-7 Breast Cancer Cells. Endocrinology 133:996-1002.

Finlay, T.H. Kadner, S.S. and Tamir, S. 1993b Protease Inhibitor Synthesis by MCF-7 Breast Cancer Cells. *in* Protease Inhibitors as Cancer Chemopreventative Agents. (W. Troll and A. Kennedy, Eds.). Plenum Press, New York. pp 141-159.

Harris, J.R., Lippman, M.E., Veronesi, U.V., Willett, W. 1992 Breast Cancer N. Engl J Med 327:319-328, 390-398, 473-480.

Hooper, N.M, Karran, E.H. and Turner A.J. 1997 Membrane protein secretases. Biochem J 321:265-279.

Kitamoto,Y., Veile,R.A., Donis-Keller,H. and Sadler,J.E. 1995 cDNA sequence and chromosomal localization of human enterokinase the proteolytic activator of trypsinogen. Biochemistry 34: 4562-4568.

Lee, D.C., Fenton, S.E., Berkowitz, E.A. and Hissong, M.A. 1995 Transforming Growth Factor $\alpha$: Expression, Regulation, and Biological Activities. Pharm. Rev. 47:51-85.

Leytus, S.P., Loeb, K.R., Hagen, F.S., Kurachi, K., Davie, E.W. (1988) A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. Biochemistry. 27:1067-1074.

Massagué, J. and Pandiella, A. 1993 Membrane-Anchored Growth Factors. Ann Rev Biochem 62:515-541.

Miyazawa, K., Shimomura,T ., Kitamura, A., Kondo, J., Morimoto, Y. and Kitamura, N. (1993) Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. J. Biol. Chem. 268:10024-10028.

Paolino-Giacobino, A., Chen, H., Peitsch, M.C., Rossier, C. and Antronarakis, S.C. 1997 Cloning of the TMPRSS2 gene, which encodes a novel serine protease with transmembrane, LDLRA, and SRCR domains and maps to 21q22.3 Genomics 44:309-320.

Rawlings ND, Barrett AJ 1994 Families of serine peptidases. Meth Enzymol 244:19-61

Sokolova, E.A., Starkova, N.N., Vorotynseva, T.I. and Zamolodchikova, T.S. 1998 A seine protease from the bovine duodenal mucosa, chymotrypsin-like duodenase. Eur J Biochem 255:501-507.

Tamir, S., Kadner, S.S., Katz, J. and Finlay, T.H. 1990 Regulation of Antitrypsin and Antichymotrypsin Synthesis by MCF-7 Breast Cancer Cell Lines. Endocrinology 127:1319-1328.

Yavelow J, Tuccillo A, Kadner SS, Katz J, Finlay TH. 1997 $\alpha_1$-Antitrypsin Blocks Release of TGF$\alpha$ from MCF-7 Human Breast Cancer Cells. J. Clin. Endo. Metabol 82:745-752.

Yu, J.X., Chao, L. and Chao, J. 1995 Molecular Cloning, tissue-specific expression and cellular localization of human prostasin mRNA. J. Biol. Chem 270:13483-13489.